

Transformation of *Escherichia coli* with large DNA molecules by electroporation

YuLing Sheng, Valeria Mancino and Bruce Birren*

Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125, USA

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ABSTRACT

We have examined bacterial electroporation with a specific interest in the transformation of large DNA, i.e. molecules >100 kb. We have used DNA from bacterial artificial chromosomes (BACs) ranging from 7 to 240 kb, as well as BAC ligation mixes containing a range of different sized molecules. The efficiency of electroporation with large DNA is strongly dependent on the strain of *Escherichia coli* used; strains which offer comparable efficiencies for 7 kb molecules differ in their uptake of 240 kb DNA by as much as 30-fold. Even with a host strain that transforms relatively well with large DNA, transformation efficiency drops dramatically with increasing size of the DNA. Molecules of 240 kb transform ~30-fold less well, on a molar basis, than molecules of 80 kb. Maximum transformation of large DNA occurs with different voltage gradients and with different time constants than are optimal for smaller DNA. This provides the opportunity to increase the yield of transformants which have taken up large DNA relative to the number incorporating smaller molecules. We have demonstrated that conditions may be selected which increase the average size of BAC clones generated by electroporation and compare the overall efficiency of each of the conditions tested.

INTRODUCTION

Transformation of bacteria with exogenous DNA has played a central role in defining the molecular events that underlie genetic phenomena (1). High efficiency transformation makes the creation of representative libraries feasible when large numbers of colonies are needed or when the source DNA is limited. Although chemical treatments of the cells have historically been the means of promoting competence to take up DNA, electroporation produces the highest efficiencies (reviewed in 2), making it the method of choice for construction of genomic libraries. Electroporation, in which cells and DNA are subjected to brief high voltage pulses, is thought to involve the transient appearance of pores (3,4), through which the DNA is driven into the cell by the electric field (5,6). Many techniques for producing the electric field have been applied (7–11). The most common method is capacitor discharge, which produces a field that decays exponentially over time (12).

The absolute efficiency of electroporation, or the number of transformants per amount of input DNA, is critical for library construction and many studies have reported procedures for increasing this efficiency. Efficiency can be influenced by factors such as temperature (13) and other electric field parameters selected by the user, including the voltage gradient, resistance and capacitance (10,14,15). In addition, qualities associated with the host cells, such as their genetic background (2), growth conditions (16) and post-pulse treatment (12), as well as the topological form and treatment of the DNA samples (17,18), will also alter the number of transformants recovered.

We are engaged in the construction of libraries containing large DNA fragments using an F factor-based vector to create bacterial artificial chromosomes (BACs) (19). In this work, partially-cut, size-selected DNA of several hundred kilobase pairs is ligated to a 7 kb vector and the ligation mix is electroporated to generate a library of clones. While a wealth of empirical observations and a strong theoretical framework exists to guide the cloning of smaller DNA fragments, the application of these procedures to larger sized DNA molecules is a recent phenomenon. Consequently, little data exist to direct cloning of large fragments in bacteria and it is not clear how well we can predict the optimal conditions for any of the steps in the procedure. For example, our lack of understanding about the uptake of large DNA molecules into bacteria has made it difficult to select conditions for the electroporation of BAC DNA. We have used the BAC system to investigate the process of electroporation, to establish which parameters are specifically important for the transformation of large DNA. BAC clones are well suited for such studies, since they are available in a variety of defined lengths, with each containing a common set of vector sequences. In addition, the blue/white color screen provides a rapid indication of the relative transformation efficiency of large versus small molecules. Finally, the F factor-derived portion of the molecules ensures that multiple BACs will not reside in a single clone, simplifying the interpretation of transformations.

MATERIALS AND METHODS

Plasmid DNA preparation

BAC mini-preps from overnight cultures were performed either in the Autogen 740 automated mini-prep system (Integrated Separation Systems, Natick, MA) using 3 ml cultures or by hand using 1.5 ml cultures. The manual preparations were accomplished using

* To whom correspondence should be addressed at present address: Whitehead Institute/MIT Center for Genome Research, 1 Kendall Square, Building 300, Cambridge, MA 02139, USA

an alkaline lysis procedure (20) with solutions I, II and III prepared according to Sambrook *et al.* (21). Cell pellets were resuspended in 100 μ l chilled solution I and placed on ice. To this was added 200 μ l freshly prepared solution II, the tubes were inverted 7–9 times to mix and replaced on ice. After adding 150 μ l solution III, the tubes were again inverted 7–9 times and immediately spun for 6 min at full speed in the microfuge at room temperature. The supernatant was poured into a new tube and the nucleic acid precipitated by addition of 1 ml room temperature ethanol. After mixing by inversion, samples were immediately spun for 6 min at full speed in the microfuge at room temperature. Precipitated DNA pellets were washed with 1 ml 70% ethanol, air dried and resuspended in 20 μ l TE (10 mM Tris, 1 mM EDTA, pH 8.0). DNA pellets from the Autogen preps were resuspended in 50 μ l. For quantitation of BAC DNA, 2 μ l mini-prepped DNA was digested with *NotI* (which excises the insert from the vector) and run on a mini gel of 0.7% agarose, along with serial dilutions of full-length, linear λ DNA and *HindIII* digests of λ DNA. Gels were stained in 1 μ g/ml ethidium bromide for 20 min. After photography, the intensities of the BAC samples were compared with those of the standards. Sample DNA was always bracketed by a range of both higher and lower concentrations of the standards and photographic exposure times were selected to avoid saturation. For electroporation, samples were diluted in water and stored at 4°C.

Preparation of competent cells

Competent cells were prepared by the procedure supplied with the electroporation device (Life Technologies, Gaithersburg, MD), with minor modifications to the protocol. A fresh overnight culture was diluted ~1:1000 for growth in SOB (without Mg^{2+}) (21). Cells were grown at 37°C with shaking at 200 r.p.m. to an OD₅₅₀ of 0.7 (never higher than 0.8, except where noted). Cells were harvested by spinning in a GSA rotor for 10 min at 5000 r.p.m. and resuspended in a volume of cold 10% sterile glycerol equal to the original culture volume. Cells were collected by spinning for 10 min at 5000 r.p.m. at 4°C. This washing and spinning procedure was then repeated, again using a volume of 10% glycerol equal to the original culture volume. After decanting the supernatant, cells were resuspended in the volume of glycerol remaining in the centrifuge bottles (~60 ml) and spun for 10 min at 7000 r.p.m. in an SS34 rotor. After decanting the supernatant, cells were resuspended in 10% glycerol, using a volume of between 2 and 2.25 ml/l initial culture. Cells were aliquoted to microfuge tubes (100–200 μ l/tube) and frozen quickly in a dry ice–ethanol bath. Cells were stored until use at –70°C. Electrocompetent cells were purchased in the case of DH5 α (Life Technologies, Gaithersburg, MD) and JS4 (BioRad Laboratories, Richmond, CA).

Electroporation

Electroporation was carried out using either a BioRad Gene Pulser or Gene Pulser II. Cuvettes with a 0.1 cm gap (BioRad Laboratories) were placed on ice to chill. Electrocompetent cells were thawed and 30 μ l added to microfuge tubes placed on ice. Two μ l DNA was added and, after mixing, the DNA and cells were transferred to the cuvette. Electroporation conditions were 100 Ω , 1.8 kV and 25 μ F, except where otherwise noted. Electroporations were performed in duplicate. After electroporation, 0.5 ml SOC was immediately added to the cuvette and the contents were then transferred to sterile glass culture tubes for 45 min growth with shaking at 37°C. For each electroporation, 2.5,

25 and 250 μ l of cells, each in duplicate, were spread on LB plates containing 12.5 μ g/ml chloramphenicol. For transformations of ligations with the BeloBAC vector, plates contained 50 μ g/ml X-gal and 25 μ g/ml IPTG. At least 24 h growth at 37°C were permitted prior to scoring colonies arising from ligations to permit the blue color to develop sufficiently.

Ligations of high molecular weight DNA

Ligations of size-selected high molecular weight mouse DNA to the *HindIII*-cut and dephosphorylated pBeloBAC vector (Shizuya *et al.*, manuscript in preparation) were carried out as described by Shizuya (19). The 7.3 kb BeloBAC vector is derived from pBAC108L (19) by incorporation of a blue/white color screen to indicate the presence of inserted DNA. Typically, ligations were carried out in volumes of 90 μ l and contained 16–20 ng vector and maintained a molar ratio of vector to insert DNA of 10:1. Prior to electroporation, ligation mixes were dialyzed for >2 h against 0.5 \times TE, containing 1 \times polyamines (1 \times polyamines is a mixture of spermine at 0.75 mM and spermidine at 0.25 mM).

Analysis of clone sizes

Mini-prepped DNA (5 μ l) was digested in a volume of 20 μ l for 1–2 h using 3 U *NotI* (New England BioLabs, Beverly, MA). Samples were run for 18 h in 1% agarose gels (SeaKem LE; FMC BioProducts, Rockland, ME) in a CHEF Mapper or CHEF DRIII pulsed field gel box (BioRad Laboratories) in 0.5 \times TBE (21) at 14°C with a voltage gradient of 6 V/cm and the switch interval ramped from 5 to 15 s. Sizes were determined by comparison with the Lowrange, Midrange I, Midrange II and λ ladder size markers of New England BioLabs.

RESULTS

Transformation of different bacterial strains with large DNA

Bacterial cells which are deficient in a number of recombination functions increase the stability of cloned DNA which contains repeated sequences. These strains exhibit a wide range in their ability to take up small DNA by electroporation (2). To test the efficiency of different *E. coli* strain transformations with large DNA molecules we used BAC DNA from clones of known sizes. Table 1 presents a comparison of transformations of DH10B, DH5 α , XL-1 Blue MR, JS4 and HB101 performed with 7.3 and 240 kb molecules and demonstrates that the ability to take up large DNA is independent of the uptake of smaller molecules. Restriction analysis and pulsed field gel electrophoresis confirmed that the size of the BACs in the transformants corresponded to the size of the intact BAC DNA used for the transformation (data not shown). DH10B, DH5 α , XL-1 Blue MR and JS4 exhibit similar transformation efficiencies with the 7.3 kb plasmid, while HB101 transforms about 5-fold less well than these other strains with 7.3 kb DNA. In contrast, uptake of the 240 kb BAC DNA shows much greater variability among these same five strains. For example, compared with DH10B, which is the most efficiently transformed of any of these strains with the large DNA, the other strains show efficiencies which vary >200-fold. The strains DH5 α , XL-1 Blue MR and JS4, which were comparable with DH10B in transformation with the 7.3 kb DNA, show a 3.8-, a 29- and a 24-fold reduction, respectively, compared with DH10B in transformation with the larger DNA. Among all the *E. coli* strains tested, the strain best transformed by large DNA

is DH10B (data on additional strains not shown). The ability of DH10B to take up 60 kb DNA has been described previously (2).

Table 1. Transformation of different *E.coli* strains with small and large DNA molecules^a

	DH10B	HB101	DH5 α	XL-1 Blue MR	JS4
7.3 kb DNA	1740	330	1390	1370	1350
240 kb DNA	1220	6	302	42	50

^aThe number of colonies obtained on transformation of different bacterial strains with two different sized DNA samples is shown (colony numbers reflect the averages from duplicate platings). DNA concentrations were not determined and thus only the relative number of transformants obtained from a single DNA sample may be compared. Electroporation conditions were 25 μ F, 200 Ω , 13 kV/cm. Transformations were performed with a range of different amounts of DNA to ensure that the number of transformants obtained was a linear function of input DNA.

Transformation efficiency and increasing DNA size

To quantitate the relative efficiency of transformation of DH10B with various sized molecules we carried out transformations with different BACs of known sizes. The efficiency of transformation with the 7.3 kb BAC vector and BACs (containing human sequences) of 80, 150 and 240 kb is shown in Table 2. The 10-fold decrease in efficiency, on a mass basis, observed for the molecules of 7.3 and 80 kb indicates that these two different sized molecules transform, on a molar basis, with nearly identical efficiency. However, when the size of the DNA is almost doubled from 80 to 150 kb, the efficiency is reduced >6-fold. A further increase in size of the DNA by a factor of 1.6 (from 150 to 240 kb) again lowers the efficiency by >10-fold. Thus there is a significant reduction in transformation efficiency with increases in size, dropping from $\sim 10^9$ colonies/ μ g for the 7.3 kb molecules, to just 10^6 colonies/ μ g for 240 kb DNA. To confirm the relative transformation efficiencies reported in Table 2 we carried out mixing experiments in which BAC DNAs of these four different sizes were combined in various proportions prior to use for transformation. After transformation, the sizes of BACs in individual colonies were analyzed to establish the frequency of transformation with each of the different size classes of DNA. The frequency of transformation for any individual BAC when mixed with BACs of different sizes reflected the transformation efficiencies shown in Table 2 (data not shown). Thus the size dependence of bacterial transformation shown in Table 2 results in an extremely strong bias which favors recovery of smaller clones when transformation is performed using mixtures of different sized DNAs.

Table 2. Efficiency of electroporation for BAC DNA of different sizes^a

Size (kb)	Efficiency
7.3	2.3×10^9
80	1.9×10^8
150	3.2×10^7
240	1.2×10^6

^aEfficiencies (colonies/ μ g) were calculated from duplicate platings. Electroporation conditions were 25 μ F, 200 Ω , 17 kV/cm. Transformations were performed with a range of different amounts of DNA to ensure that the number of transformants obtained was a linear function of input DNA.

Electric field strength effects on large DNA transformation

A bias in the transformation against large DNA molecules would obviously have undesirable consequences for generating BAC libraries from a ligation mix containing a heterogeneous population of DNAs. We therefore examined some of the electroporation parameters to determine if conditions existed which would improve transformation by large DNA. Transformations were performed with different sized BACs using a variety of electric field strengths. The efficiencies resulting from transformation with BACs at field strengths ranging from 5 to 25 kV/cm are shown in Table 3. Transformation of *E.coli* with small plasmids has been shown to be optimal at field strengths of ~ 16 – 18 kV/cm (12). We see the maximum efficiency for the 7.3 kb molecule occurring at 17 kV/cm. At 17 kV/cm, 1.4-fold and nearly 2-fold more colonies are obtained than at either 13 kV/cm or 21 kV/cm respectively. However, all the larger molecules tested, the 80, 150 and 240 kb BACs, show maximum transformation at a lower field strength, namely 13 kV/cm. Transformation of the 150 and 240 kb molecules was three times more effective at 13 kV/cm than at 17 kV/cm, while the 80 kb DNA was 2.3-fold more effective at 13 kV/cm.

The results of the transformations at each field strength shown in Table 3 are plotted as a percent of the maximum efficiency obtained for each size of DNA in Figure 1. Compared with transformation of the 7.3 kb DNA, all points on these curves for the 80, 150 and 240 kb DNA are shifted toward lower voltages. At 18 kV/cm, which produces the peak efficiency for transformation of small DNA, the large DNAs are transformed at only $\sim 30\%$ of their maximum. The important implication for BAC cloning from Table 3 and Figure 1 is that lower field strengths offer not only the highest absolute efficiency for transformation of large DNA, but also increase the efficiency of large DNA transformation relative to smaller DNA. Note that at 9 kV/cm the 240 kb DNA transformed at 73% of its maximum level, but transformation with the 7.3 kb DNA produced only 7% of its maximum. Thus for BAC cloning, where the relative transformation efficiency of large and small molecules determines the composition of the library, conditions of low field strength may prove useful.

Table 3. Transformation efficiency of different sized DNA by electroporation at different voltages^a

Voltage gradient (kV/cm)	Efficiency 7.3 kb	80 kb	150 kb	240 kb
21	1.2×10^9	3.2×10^7	2.1×10^6	2.0×10^5
17	2.3×10^9	1.9×10^8	3.2×10^7	1.2×10^6
13	1.6×10^9	4.4×10^8	1.0×10^8	3.7×10^6
9	1.6×10^8	1.5×10^8	3.6×10^7	2.7×10^6
5	$<10^6$	8.4×10^5	7.0×10^5	5.0×10^4

^aEfficiencies (colonies/ μ g) were calculated from duplicate platings of different amounts of transformed cells. Electroporation conditions were 25 μ F, 200 Ω , with the indicated voltage gradients.

Transformation of large DNA using different resistances

Next we examined whether resistances which result in optimal electroporation would differ for large and small DNA when using capacitor discharge. Changes in resistance alter the length of time over which the electric pulse is delivered. This time period, referred to as the time constant (t), is a function of the resistance and capacitance selected [t (s) = R (ohms) \times C (farads)]. The results of

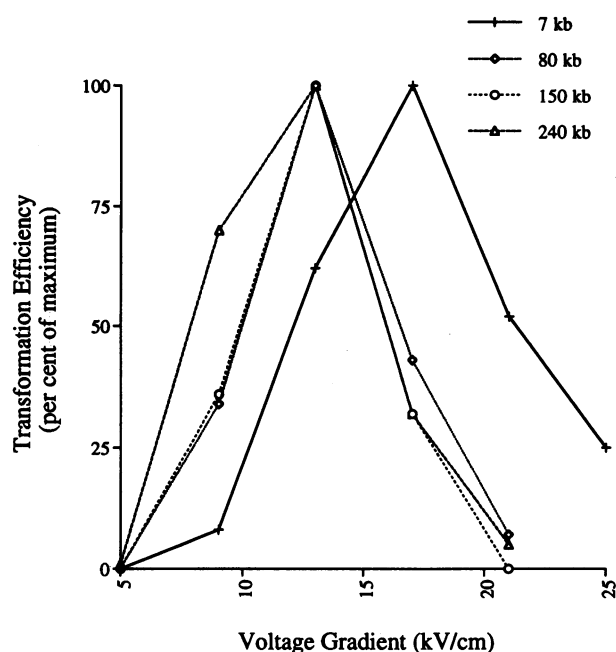


Figure 1. Transformation efficiency as a function of voltage gradient for DNA of different sizes. Data from Table 1 is plotted as the percent of the maximum efficiency attained for each of the different sized molecules used: 7.3 (+), 80 (o), 150 (o) and 240 kb (Δ).

transformations with the 7.3 and 240 kb BAC clones carried out at 100, 200 and 400 Ω using voltage gradients of 13 kV/cm are shown in Table 4. These two different sized DNAs show peak transformation efficiencies at different resistances; the smaller DNA transforms best using 200 Ω , while the large DNA transforms best at 100 Ω . Specifically, at a voltage gradient of 13 kV/cm, the transformation efficiency of the 240 kb DNA at 100 Ω ($t = 2.4$ ms) is ~3- and 7-fold higher respectively than that obtained at 200 ($t = 4.6$ ms) or 400 Ω ($t = 8.8$ ms). In contrast, the 7 kb DNA at 200 Ω gave rise to nearly 3-fold more colonies than at 100 Ω , and 2-fold more colonies than transformation at 400 Ω . Transformations performed using 50 Ω produced lower efficiencies for each of these DNAs (data not shown). When a higher voltage gradient of 18 kV/cm was used, the two different sized DNAs also exhibited maximum transformation efficiency at the different resistances, though as expected, the absolute number of colonies obtained with the 240 kb DNA was lower (data not shown).

Table 4. Electroporation of small and large DNA using different resistances^a

Size (kb)	100 Ω	200 Ω	400 Ω
7.3	173	508	267
240	945	318	127

^aColony numbers shown are the average of two separate electroporations. DNA (2 μ l) was electroporated with 25 μ l cells using settings of 25 μ F and 13 kV/cm. Time constants (t) obtained were: 100 Ω , 2.4 ms; 200 Ω , 4.6 ms; 400 Ω , 9.0 ms. DNA concentrations were not determined and thus only the relative number of transformants obtained from a single DNA sample may be compared. Transformations were performed with a range of different amounts of DNA to ensure that the number of transformants obtained was a linear function of input DNA.

Transformation with BAC ligation mixes using different electroporation conditions

The above results indicate that optimal electroporation with large BAC DNA occurs under significantly different conditions than those traditionally used for smaller DNA. However, these results were obtained with DNA purified by mini-preps of existing BAC clones. The relevance of such experiments to the process of clone construction cannot be assumed, given the differences between mini-prepped DNA and a ligation mix. We therefore used ligation mixes of the BeloBAC vector with large (≥ 100 kb) mouse DNA to test conditions for electroporation. Data from three separate experiments in which aliquots from ligation reactions were transformed using different field strengths are shown in Table 5. The efficiency of cloning, i.e. the yield of clones/ μ g insert DNA, is influenced by properties associated with each preparation of high molecular weight DNA and varied considerably between these three experiments. However, the BeloBAC vector makes use of the α complementation of β -galactosidase to indicate disruption of the cloning site by conversion of blue colony color to white (22). In transformations with a ligation mix, blue colonies arise from uptake of the 7.3 kb vector, while white colonies indicate the presence of inserted DNA or other changes to the cloning site. The ratio of white to blue colonies thus represents a first approximation of the cloning efficiency, since blue colonies indicate the background of vector without inserts.

In experiment 1 a single ligation was electroporated with field strengths of either 9, 13, 17 or 21 kV/cm. Consistent with the results obtained using mini-prepped DNA, the highest total number of colonies was obtained at 13 kV/cm, while the most extreme field strengths, i.e. 9 and 21 kV/cm, produced the lowest number of total colonies (Table 5). However, the fraction of white colonies progressively rose as the field strength was decreased, such that although less than half the total colonies were obtained at 9 compared with 13 kV/cm, the fraction of white colonies doubled at the lower field strength. Since white colony color does not indicate the size of inserted DNA, we analyzed mini-prepped BAC DNA from individual white colonies obtained under each of these conditions to determine whether electroporation conditions could affect the size of the population of BACs arising from the transformation. The restriction enzyme *NotI* cuts on each side of the cloning site, excising the inserts from the BAC vector and permitting determination of insert sizes via pulsed field gel electrophoresis. The *NotI* digestions of these clones (Fig. 2) reveal that the proportion of white colonies which contain large inserts increases as the field strength is reduced. Only three of the 16 white colonies obtained by electroporation at 17 kV/cm contained inserts larger than the vector itself, but this fraction nearly doubled when the field strength was lowered to 13 kV/cm, and nearly doubled again at 9 kV/cm. This is not surprising, since the white colonies with no or very small inserts should, just like the blue colonies, be transformed relatively less well than clones with larger inserts at the lower field strengths. In these gels, lanes without visible insert bands are presumed to contain very small fragments which are not detected and lanes without a band corresponding to the vector contain very small deleted forms of the vector without insert. Although the number of clones analyzed was small, it is intriguing that the average size of the clones generated from the ligation mix appears to increase as the field strength is reduced (Table 5). The increase in average size of the clones obtained with 13 kV/cm (111 ± 17.5 kb, average \pm SEM) compared with those produced at 17

kV/cm (93 ± 23.1 kb) is in keeping with the observation that for the larger mini-prepped BAC DNA; 13 kV/cm produced optimum efficiency (Fig. 1). However, the average size of clones generated at 9 kV/cm (139 ± 15.0 kb) was even higher than that for the clones produced with 13 kV/cm. This was a surprise, given that mini-prepped DNA from large BAC clones transformed less well at 9 than at 13 kV/cm.

We therefore repeated this experiment with different ligation mixtures to determine whether the increase in the fraction of white clones, as well the increase in the average size of the clones, produced at 9 compared with 13 kV/cm was reproducible. Experiments 2 and 3 used high molecular weight DNA which was cloned with much higher efficiency than that used in experiment 1. We presume that less damage was incurred during the preparation and isolation of the DNA used in experiments 2 and 3, resulting in higher cloning efficiencies. Consequently, there was a much higher proportion of white colonies from the ligations in experiments 2 and 3 and virtually all white colonies contained large inserts. However, the same phenomenon was observed in both cases, namely when the field strength was lowered, the fraction of white colonies rose. In experiment 2 a reduction of the field strength from 13 to 9 kV/cm increased the proportion of white colonies from 79 to 85% and in experiment 3 a reduction in field strength from 12.5 to 9 kV/cm raised the fraction of white colonies from 45 to 67%. In both these experiments the average size of the cloned DNA was also higher when the lower of the two field strengths was used for the electroporation. In experiment 2 the average size increased from 98 ± 5.8 to 127 ± 8.9 kb and in experiment 3 from 116 ± 8.6 to 132 ± 8.2 kb.

Absolute cloning efficiency resulting from different electroporation conditions

The experiments presented in Table 5 demonstrate that changes

in the electroporation conditions can alter the characteristics of a library resulting from a single ligation, including the frequency of 'empty' clones lacking inserts and the average size of the clones. Use of the higher voltage gradients routinely employed for small plasmids (e.g. 18 kV/cm) will severely impair efforts to generate large fragment libraries by electroporation. However, the choice of whether to electroporate at 13 or 9 kV/cm calls for an important distinction to be made between the average size of the clones and the absolute cloning efficiency provided by specific electroporation conditions. Table 5 also shows the genomic coverage represented by the clones generated in the different transformations. The term coverage refers to the product of the number of clones with inserts and the average size of those inserts. Despite the increase in the average size of the clones produced at lower field strengths, the resulting decline in absolute efficiency means that the amount of DNA that can be cloned using those conditions is reduced at the lower field strength. For example, in experiment 2, although the average clone size rose from 98 to 127 kb, the coverage provided by the clones generated was reduced nearly 4-fold, because of the 5.6-fold drop in the number of clones recovered at the lower voltage. In experiment 3, while the average size rose from 116 to 132 kb, 2-fold fewer clones were produced, reducing the overall coverage 1.7-fold. Thus the trade-off for the increased average clone size that accompanies electroporation at the very low field strength of 9 kV/cm is a reduction in overall cloning efficiency. In our recent construction of a mouse BAC library a high cloning efficiency permitted us to routinely use 9 kV/cm for all electroporations (Mancino *et al.*, not shown), which is approximately half the field strength previously recommended for transformation of smaller DNA (12).

Table 5. Transformation of BAC ligation mixtures by electroporation at different field strengths

Field strength (kV/cm)	Total no. of colonies	No. of white colonies/ no. of blue colonies (% white)	White colonies with inserts/ white colonies analyzed	Insert size (kb) ($n \pm \text{SEM}$)	Coverage (Mb)
Experiment 1					
21	4746	7/4739 (1.5%)	4/7 (57%)	$22 \pm (4.5)$	0.09
17	5213	16/5197 (3.1%)	3/16 (19%)	$93 \pm (23.1)$	0.28
13	8558	29/8529 (3.4%)	7/20 (35%)	$111 \pm (17.5)$	1.1
9	3238	22/3216 (6.8%)	12/20 (60%)	$139 \pm (15.0)$	1.8
Experiment 2					
13	10 029	7904/2125 (79%)	19/20 (95%)	$98 \pm (5.8)$	736
9	1755	1485/270 (85%)	20/20 (100%)	$127 \pm (8.9)$	189
Experiment 3					
12.5	14 900	6700/8200 (45%)	19/19 (100%)	$116 \pm (8.6)$	777
9	8396	3373/5023 (67%)	20/20 (100%)	$132 \pm (8.2)$	445

Three different ligation mixtures containing different preparations of size-selected high molecular weight mouse DNA and the BeloBAC vector were transformed using different field strengths. Multiple aliquots were electroporated from each ligation under the different conditions and the entire quantity of transformed cells were plated from each electroporation. After electroporation, aliquots were spread on 150 mm dishes, with the total number of colonies (white + blue) ranging up to 1000 on any dish. The percentage of white colonies of the total number of colonies produced is shown. Random white colonies from each electroporation were analyzed for the presence and size of insert DNA, by digestion of mini-prep DNA with *NotI* and PFGE analysis, as in Figure 2. The number of white colonies with inserts reflects only those clones which contained inserts >4 kb. The percentage shown for white colonies which were found to contain inserts is also shown for each condition. The average size and the standard error of the mean (SEM) for these sizes is shown for each electroporation. Calculations of average size, which include clones containing inserts smaller than 4 kb, yield the same trend of voltage dependence, though with smaller average sizes. Coverage refers to the total amount of genomic DNA cloned under each condition and is calculated from the total number of white colonies, the proportion of white colonies with inserts and the average size of those inserts. Experiment 1 was performed using a resistance of 200 Ω , while experiments 2 and 3 were performed using 100 Ω .

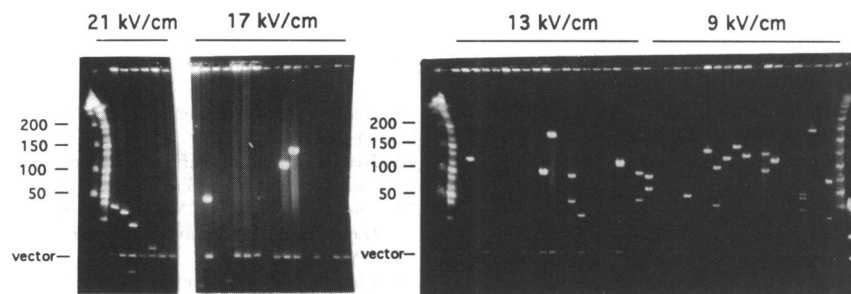


Figure 2. Transformants arising from electroporation of a ligation mix at different voltage gradients. A single ligation mix was used for multiple electroporations with a voltage gradient of 9, 13, 17 or 21 kV/cm. Mini-prepped DNA from resulting white colonies was digested with *NotI* and analyzed by pulsed field gel electrophoresis. The sizes associated with the lambda ladder size markers (the far left lane of each gel) are indicated in kb. In addition, each gel contained the Midrange I, Midrange II and Lowrange size marker of New England Biolabs. The common band of 7.3 kb is that of the BeloBAC vector.

DISCUSSION

Role of the host cell

The efficiency of bacterial transformation using the most common method of electroporation is strongly size related. We have shown an ~20-fold reduction in the transformation efficiency, on a molar basis, when the size of transformed BACs is increased from 150 to 240 kb. For strain DH10B diminished transformation efficiency is not apparent until DNA size is >80 kb. As our ability to prepare and handle DNA of defined sizes in this range is a recent development, it is not surprising that the size dependence of transformation for molecules in this size range is not well understood. For example, although it has been reported that strains such as DH10B, which contain a mutation in the *deoR* gene, exhibit higher transformation efficiencies with 60 kb DNA (2), the basis for this is not known. This mutant was originally selected for its ability to grow with inosine as the sole carbon source; the relationship of this phenotype to properties of electroporation has not been established. The fact that uptake of large DNA is so strongly governed by the genetic background of the host suggests that there may be other mutations which could similarly influence large DNA uptake. At the present, only *deoR* mutants are known to take up large DNA sufficiently well to consider them as hosts for large insert library construction. Remarkably, the transformation efficiency of DH10B with molecules as large as 80 kb was comparable, on a molar basis, with that for 7.3 kb molecules. This is not the case with other strains tested (data not shown). The fact that such high transformation efficiencies can be obtained for molecules up to at least 80 kb indicates that for DH10B electroporation can equal or exceed the efficiency of transformation produced by *in vitro* packaging to produce infectious P1 phage particles (23).

Different optimal conditions for electroporation of large and small DNA

Within the limited number of parameters we have examined, differences exist in the optimum conditions for transformation by large, as opposed to small, DNA molecules. Electroporation consists of at least three distinct processes: formation of pores, entry of the DNA into the cell and recovery of the cells. Each of these steps may be size sensitive. The molecules under consideration have very long contour lengths relative to the size of the cells. If open circular DNA is fed through the pore doubled back

on itself, then DNA molecules of vastly different sizes would have the same dimensions in the pore, but would require different lengths of time to complete their entry. Pulse regimens that employ a brief initial high voltage pulse to open the pore, followed by a longer period of lower voltage to allow longer molecules more time for entry (6) may prove even more effective for transformation of large DNA than the single-pulse regimen we have used. The difference in optimum field strength for large and small DNA could reflect damage to the large DNA at the higher field strengths. It has been shown that λ DNA can be denatured by exposure to the high field strengths used for electroporation (24) and that although the use of higher field strengths results in larger amounts of DNA being transferred into cells, the ability of this DNA to function, as measured by transformation efficiency, shows a maximum at lower field strengths (17). The conditions we now employ for transformation of molecules >50 kb are: 9 or 12.5 kV/cm, 100 Ω , 25 μ F. Elucidation of the actual process by which large DNA enters the cell should significantly enhance our ability to further increase transformation frequencies.

Electroporation conditions and library construction

The strong size dependence of electroporation has several implications for the process of large insert library construction and manipulation. Since library construction involves transformation with a mixture of different sized molecules, electroporation introduces bias towards recovery of clones originating from the smallest molecules in the mixture. Insert DNA is often prepared using pulsed field gel electrophoresis, but these gels are extremely sensitive to overloading, which promotes co-migration of small DNA with molecules that are much larger. Thus careful size selection of DNA which is prepared at an appropriate concentration is needed to ensure that only a narrow size range of molecules will be present for the transformation step. If large DNA molecules have been purified with the intent of generating large clones, these molecules will transform with the decreased efficiency associated with their large insert size. We routinely see a dramatic decrease in cloning efficiency when DNAs which differ by rather modest increments of size above 80 kb are used (Mancino and Birren, unpublished observations).

While the overall BAC cloning efficiency is the result of a series of complex events, the size dependence of the BAC cloning efficiency that we see strongly parallels the size dependence of

transformation efficiencies obtained with purified BACs of known sizes. Further, we have demonstrated the ability to alter the make-up of libraries simply by modulating the electroporation conditions. For example, with the same ligation we were able to increase the average size of the clones produced from 93 to 139 kb and double the number of clones which contained inserts (Table 5, experiment 1). Condensing the ligated DNA prior to transformation by introduction of supercoiling may also increase transformation efficiency. The difficulty of quantitating pure populations of supercoiled and open circular large DNA molecules has so far prevented a precise evaluation of the relative transformation efficiency of these two species.

Finally, it is important that large insert libraries be maintained as frozen bacteria and not as DNA stocks. First, we observe a rapid decrease in the transforming ability of mini-prepped BAC DNA upon storage at 4°C, which is much more pronounced than for smaller molecules. This decrease occurs even more rapidly with cycles of freezing and thawing of the BAC samples, while small molecules are relatively unaffected by this process (data not shown). More seriously, if even a small proportion of the DNA in a sample consists of deleted molecules, these will transform more efficiently than the original larger DNA and thus will be preferentially recovered. Transformation itself does not appear to induce rearrangements or deletions for these clones (25; Shizuya, unpublished results), however, it introduces a strong selection for the rare deletions that do occur.

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